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REVIEW

Ischemia/reperfusion injury and cardioprotective mechanisms: Role of mitochondria and reactive oxygen species

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Abstract

Reperfusion therapy must be applied as soon as possible to attenuate the ischemic insult of acute myocardial infarction (AMI). However reperfusion is responsible for additional myocardial damage, which likely involves opening of the mitochondrial permeability transition pore (mPTP). In reperfusion injury, mitochondrial damage is a determining factor in causing loss of cardiomyocyte function and viability. Major mechanisms of mitochondrial dysfunction include the long lasting opening of mPTPs and the oxidative stress resulting from formation of reactive oxygen species (ROS). Several signaling cardioprotective pathways are activated by stimuli such as preconditioning and postconditioning, obtained with brief intermittent ischemia or with pharmacological agents. These pathways converge on a common target, the mitochondria, to preserve their function after ischemia/reperfusion. The present review discusses the role of mitochondria in cardioprotection, especially the involvement of adenosine triphosphate-dependent potassium channels, ROS signaling, and the mPTP. Ischemic postconditioning has emerged as a new way to target the mitochondria, and to drastically reduce lethal reperfusion injury. Several clinical studies using ischemic postconditioning during angioplasty now support its protective effects, and an interesting alternative is pharmacological postconditioning. In fact ischemic postconditioning and the mPTP desensitizer, cyclosporine A, have been shown to induce comparable protection in AMI patients.

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Key words: Adenosine triphosphate-dependent potassium channels; Cardioprotection; Ischemia-reperfusion injury; Mitochondrial permeability transition pore; Reactive oxygen species

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INTRODUCTION

Acute myocardial infarction (AMI) is responsible for the death of millions of persons worldwide each year, with a mortality rate of about 10%, and is the leading cause of chronic heart failure[1]. Notwithstanding, marked improvements in the strategy to reduce infarct size and to reduce all manifestations of postischemic injury, with subsequent improvement in prognosis, have been developed in recent years. Although early reperfusion is the

only way to salvage an ischemic organ, during the crucial early moments of reperfusion, significant reversible and irreversible organ damage is initiated, and is referred to as reperfusion injury. Reperfusion injury include arrhythmias, transient mechanical dysfunction of the heart or "myocardial stunning", microvascular injury and "no-reflow", as well as inflammatory responses. In reperfusion, cell death can occur due to apoptosis, necrosis, and autophagy[2-8]. Given that recent data indicate that the different forms of cell death are probably interrelated[6,7], a better strategy to develop cardioprotective agents is not to define the mode of cell death and its proportion occurring during ischemia/reperfusion, but to identify mediators active in all forms of cell death. In this context, the variation in mitochondrial membrane permeability appears to be one of the major regulators of all forms of cell death.

REPERFUSION INJURY: CENTRAL ROLE OF MITOCHONDRIA

During normal perfusion mitochondria generate adenosine triphosphate (ATP), consume large amounts of O_2 and contribute to a balanced generation and scavenging of reactive oxygen species (ROS). Mitochondria are also involved in cellular ion homeostasis, including calcium homeostasis.

During ischemia, the lack of of O_2 inhibits electron flow, and myocardial ATP utilization becomes inefficient. The proton-translocating F_0F_1ATP synthase, which normally produces ATP, switches into reverse mode, i.e. becomes an F_0F_1ATPase, and consumes ATP to pump protons from the matrix into the intermembrane space[9,10]. In prolonged ischemia, Na^+/K^+ ATPase is inhibited (because of the drop in ATP levels) and the intracellular acidification (induced by lactate production and the hydrolysis of ATP) activates the Na^+/H^+ exchanger (NHE), i.e. the cell tries to restore the intracellular pH; the resulting increase in intracellular Na^+ concentration activates the Na^+/Ca^{2+} exchanger (NCE), which lead to Ca^{2+} overload. Elevated cytosolic Ca^{2+} concentrations may contribute to cellular damage by activation of degrading enzymes such as nucleases, phospholipases and proteases culminating in the destruction of the membrane integrity and leading to cell death if the ischemic period is of sufficient duration[11,12].

At reperfusion, intracellular and mitochondrial events such as Ca^{2+} overload, inadequate resynthesis of ATP, loss of membrane phospholipids, low production of nitric oxide ($NO^{\bullet}$) and oxidative stress by ROS contribute to reperfusion injury[13-16]. Yet, when an increase in ATP concentration occurs, it paradoxically contributes to reperfusion injury, leading to hyper-contracture of cardiomyocytes, membrane disruption and subsequent band necrosis[16,17]. Clearly the recovery of pH, oxidative stress and Ca^{2+} overload can induce the abrupt opening of the mitochondrial permeability transition pores (mPTP), a large conductance pore in the inner mitochondrial membrane (IMM), which strongly contributes to cardiomyocyte hyper-contracture, apoptosis and necrosis[18-22].

MPTP

This pore is a high conductance megachannel, which builds up at the contact sites between the mitochondrial outer and inner membranes. When the mPTP is formed, it permits communication between the cytoplasm and mitochondrial matrix[23]. The molecular identity of the protein(s) forming this pore is still unknown. It has been suggested that the mPTP is formed by the voltage-dependent anion channel in the outer mitochondrial membrane (OMM), the adenine nucleotide transporter in the IMM, and cyclophilin D (Cyp D) in the matrix of mitochondria. mPTP opening seems to be facilitated by binding of the matrix protein Cyp D to the IMM in a process regulated by both Ca^{2+} and inorganic phosphate (Pi)[19] (Figure 1). However, even experiments with transgenic mice in each of the putative components of mPTP reached controversial results[24-31].

mPTP opening: The oxidative opening of mPTP is central in reperfusion injury (Figure 2A). Many studies have indeed revealed an important contribution of mPTP opening and have correlated cell death with the release of cytochrome c (Cyt c) after Bax and enhanced ROS levels[22,32-35].

Importantly, the opening probability of mPTP of the de-energized mitochondria is drastically reduced below pH 7.4, a condition occurring during sufficiently prolonged ischemia[36]. Low pH also reduces mitochondrial calcium uptake and favors calcium extrusion from the mitochondrial matrix, due to the activation of mitochondrial NHE and subsequent NCE[37]. However, low pH may activate uncoupling proteins (UCPs), IMM carriers of H^+ that uncouple ATP synthesis from oxygen consumption[38]. For the role of UCPs in cardioprotection see below[39-41]. Low pH may also inhibit glycolysis and pyruvate production, resulting in a slower feeding of the respiratory complex chain. Therefore low pH mainly prevents mPTP opening in ischemia (Figure 1A).

On reperfusion, quite different conditions are created depending on whether or not the mitochondrial membrane potential rapidly recovers. In the case of energized respiring mitochondria, a low pH can stimulate Pi uptake increasing its intra-mitochondrial content, thus acting as an mPTP opener[42]. In contrast, in the presence of mitochondrial membrane depolarization, long-lasting opening of the mPTP take places when rapid normalization of tissue pH occurs in the presence of Ca^{2+} overload, Pi, ROS formation, and/or lower levels of $NO^{\bullet}$[32,43-46]. The latter condition (i.e. membrane depolarization and rapid pH normalization) is the more common scenario upon abrupt reperfusion. In fact, the probability of this pore being open is facilitated by several factors including high pH, Ca^{2+} overload, and burst of ROS at the onset of reperfusion. Apart from its direct action on mitochondria, the opening effect of Ca^{2+} is also due to indirect effects, such as phospholipase A_2 and calpain activation[47-50] (Figure 1A).

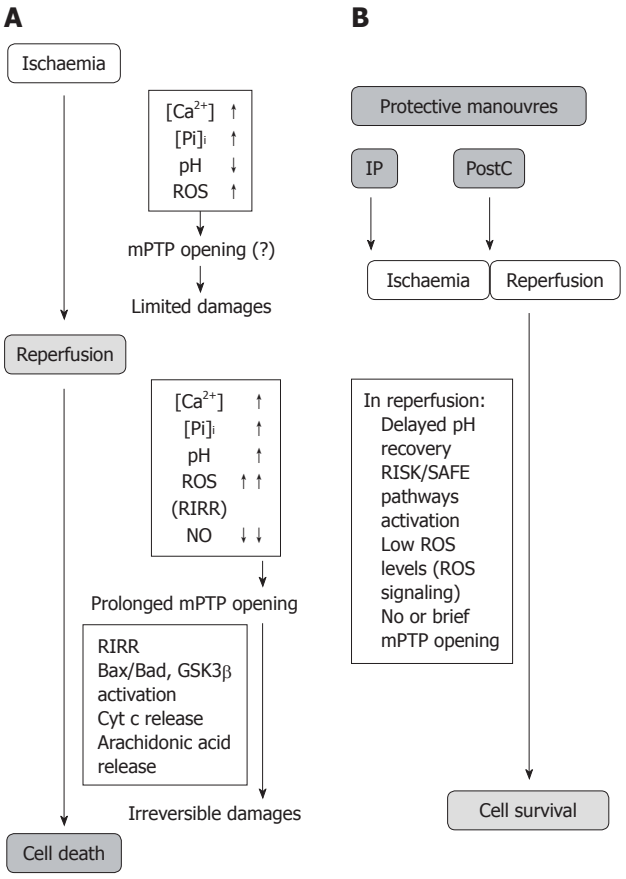

Figure 1 Flowchart depicting the variations of pH, ROS and mPTP opening during ischaemia and reperfusion phases in the control hearts, and in hearts protected by preconditioning or postconditioning. A: In the control hearts reactive oxygen species (ROS) production slightly increases during the initial part of ischaemia until the O_2 is exhausted. Then sharply increases in reperfusion. Formation of mitochondrial permeability transition pores (mPTP) had been limited during ischaemia by the low pH despite increased cellular levels of ROS, Ca^{2+} and Pi overload. But as pH returns to its baseline level and ROS formation increases prolonged opening occurs. The limited damages occurring during ischaemia are exacerbated by the prolonged mPTP opening which mediates irreversible cell damages in reperfusion. The opening effect, besides Ca^{2+} overload, is also due to indirect effects, such as phospholipase A2 (PLA2) and calpain activations and consequent arachidonic acid release after membrane phospholipids degradation. A part membrane depolarisation also inorganic phosphate (Pi), lower levels of nitric oxide (NO) contribute to mPTP opening. Other factors which regulate pore formation are Bcl-2–associated X protein (Bax)/Bcl-2-associated death promoter (Bad), B-cell lymphoma 2 (Bcl-2) and glycogen synthase kinase 3 β (GSK-3β). Pore opening leads to cell-death through the release of pro-apoptotic factors as cytochrome c (Cyt c) and via ROS-induced ROS release (RIRR); B: The pre/postconditioned hearts are characterized by delayed pH recovery, ROS signalling and activation of protective pathways (e.g. Reperfusion Injury Salvage Kinases (RISK)/Survivor Activating Factor Enhancement (SAFE). These conditions contribute to reduce mPTP opening and consequent cell death limitation. The details of the protective signalling (RISK/SAFE) can be seen in Figure 2 and Figure 3. For further explanations see the text.

Consequences of mPTP opening: Depending on the complex balance between cellular inducers and antagonists, mPTP can undergo transient or intermediate/long-lasting opening[51]. mPTP opening of short duration is likely to generate reversible cellular changes, so that this transient opening has been suggested to be involved in physiological processes and cardioprotection, such as intracellular NAD^+ traffic, and transient formation of ROS (see also below)[52-55]. Actually, the transient increase in the opening probability of mPTP is involved in ROS-dependent triggering of cardioprotection by preconditioning[56,57] (see below).

While a transient/intermediate pore opening may or may not lead to apoptosis, long-lasting pore formation is followed by profound alterations of cellular bioenergetics that are considered irreversible; it results in increased mitochondrial permeability to ions and solutes with molecular weights of up to 1.5 kDa, matrix swelling and loss of critical electrochemical gradients. In this condition the F_0F_1ATPase actively hydrolyzes rather than synthesizes ATP, leading to inevitable cell death[11,12,33,58,59]. Actually, the mandatory consequence of long-lasting mPTP opening is the collapse of mitochondrial membrane potential. This is rapidly followed by ATP and NAD^+ depletion, mitochondrial release of accumulated Ca^{2+}, matrix swelling and rupture of the OMM leading to loss of pyridine nucleotides and release of pro-apoptotic factors such as Cyt c, which triggers apoptosis and thus also inhibits electron flow through the electron transport chain[11,19,20,32,60-62]. Many have postulated that long-lasting mPTP formation is the event that leads to irreversible changes in cellular function and cell death[13,59,62,63]. Di Lisa *et al*[62] were among the first to observe that addition of Ca^{2+} to mitochondria causes organelle swelling and profound decreases in NAD^+ content.

At least two mechanisms which are not mutually exclusive have been proposed to explain mitochondrial membrane permeabilization and apoptosis. Apart the mPTP, which involves the participation of both the IMM and the OMM, a mechanism of mitochondrial death which involves only the OMM and the formation of channels across the membrane has been described. Although there is controversy concerning the structure, the regulation and the definite role of these two putative different channels, strong evidence indicates that proteins of the Bcl-2 family may contribute to both mechanisms[64,65].

Prevention of prolonged mPTP opening: All these opening factors are counteracted by "physiological" mPTP antagonists, such as adenine nucleotides (mainly ADP), elevated concentrations of protons (i.e. pH below 7.4), increased mitochondrial membrane potential, and magnesium ions, as well as by physiological levels of nitric oxide[19,66]. The pore is rapidly closed if Ca^{2+} is chelated[19,60]. Promotion of mPTP opening is also prevented by some drugs, including cyclosporine A (CsA), which at nM concentrations is a mPTP desensitizer[36,58,67]. Notably, in the absence of Pi the desensitizing effects of CsA are no longer present[58,67].

Since mPTP formation is likely to be a causative event in reperfusion injury and a major proportion of cell death results from mPTP formation, it is not a surprise that cardioprotective strategies demonstrated that inhibition of mPTP is the end-effector of cardioprotection (Figures 1B and 2). In fact the importance of mPTP closure as a target for myocardial protection has been described in several studies[58,68-70]. The mechanisms of cardioprotec-

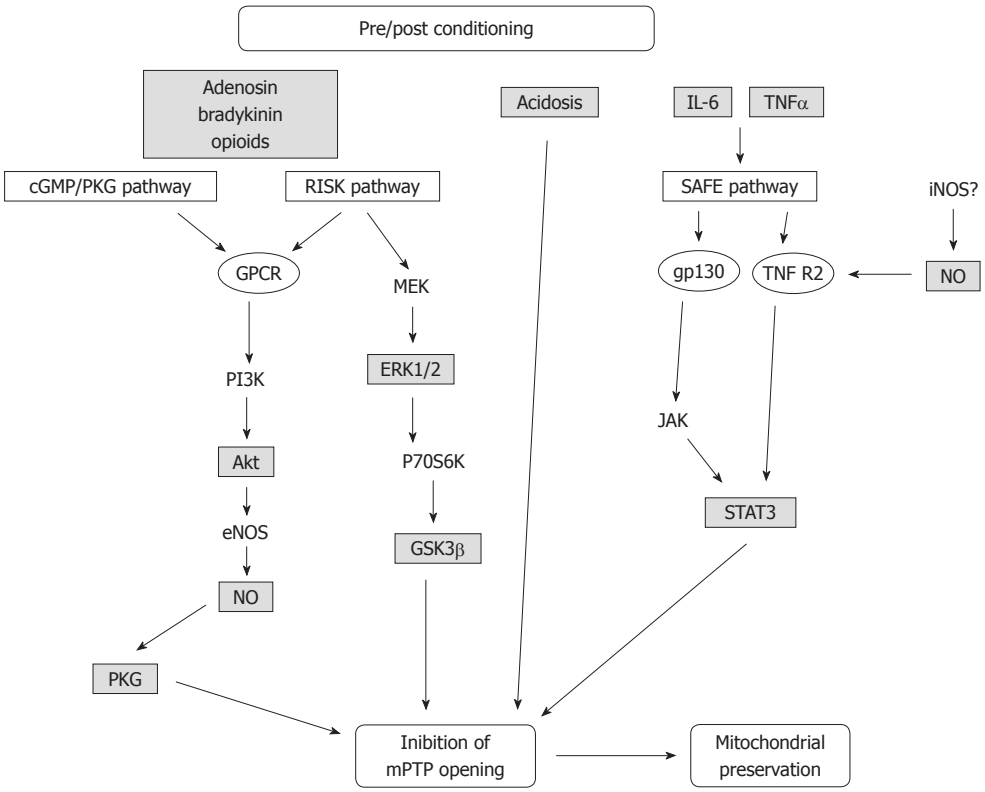

Figure 2 Flowchart depicting the main factors involved in cardioprotective pathways triggered by pre and postconditioning. Activation of cell-surface receptors in response to an ischaemic conditioning stimulus recruits cGMP/PKG, RISK and SAFE pathways. In particular, iNOS seems to be involved in SAFE pathway[77]. These signal transduction pathways, together with acidosis, activated at the time of reperfusion will crosstalk and will terminate on mitochondria to activate protective pathways. Akt: Serine/threonine protein kinase; cGMP/PKG: Cyclic guanosin monophosphate/protein kinase G; eNOS: Endothelial NO synthase; ERK1/2: Extracellular regulated kinase 1/2; gp130: Glycoprotein 130; GPCR: G-protein-coupled receptor; GSK3β: Glycogen synthase kinase 3 β; IL-6: Interleukin 6; iNOS: Inducible NO synthase; JAK: Janus kinase; MEK: Mitogen-activated protein kinase kinase; mPTP: Mitochondrial permeability transition pore; NO: Nitric oxide; P70S6K: p70 ribosomal S6 protein kinase; PI3K: Phosphoinositide 3-kinase; PKG: Protein kinase G; RISK: Reperfusion injury salvage kinases; SAFE: Survivor activating factor enhancement; STAT-3: Signal transducer and activator of transcription 3; TNFα: Tumour necrosis factor α; TNF-R2: Tumour necrosis factor receptor 2.

tion and mPTP closure in reperfusion are described in the following section.

CARDIOPROTECTIVE STRATEGIES TARGETING MITOCHONDRIA

Lethal reperfusion injury appears to represent from 20 to 70% of the total amount of irreversible myocardial damage according to the studied species and therefore constitutes a major therapeutic target[2,71-75].

Preconditioning and postconditioning

Over the last decades ischemic preconditioning and postconditioning have been recognized as protective phenomena and have been confirmed in humans; they share certain signaling elements in experimental analyses[2,76-78] (Figure 2). In 1986, Murry *et al*[79] reported that four 5 min circumflex occlusions, each separated by 5 min of reperfusion, followed by a sustained 40 min occlusion (index ischemia = infarcting ischemia) in the dog heart dramatically attenuated ischemia/reperfusion injury. This phenomenon was named ischemic Preconditioning (PreC). In 2003, Zhao *et al*[73] reported that three episodes of 30 s of reperfusion/30 s of ischemia performed immediately after index ischemia (60 min coronary occlusion) in the dog heart drastically attenuated reperfusion injury. This phenomenon was named ischemic Postconditioning (PostC). It was soon clear that the later the application of the first postconditioning ischemia, the lower the protection.

The recognition of the ischemic PostC phenomenon put an end to any discussion on the existence of reperfusion injury[80]. The term "PostC" has also highlighted the importance of intervening at the beginning of myocardial reperfusion to protect the post-ischemic heart; a clinically more relevant time-point for intervention in patients presenting with an AMI. As such, its clinical application has been rapid for both ST-elevation AMI patients undergoing primary percutaneous coronary intervention (PCI)[81,82] and for patients undergoing on-pump cardiac surgery[83] (see also below).

The protective effects observed with PostC are comparable to those observed with the powerful PreC[2,73,84,85]. In fact, PostC may reduce apoptosis, necrosis, and endothelial dysfunction/activation, thus leading to a reduced endothelium/leukocyte interaction and to reduced ROS inflammatory formation[5,73]. PostC also reduces the incidence of reperfusion arrhythmias[86-90].

Intensive investigation of the signaling pathways underlying PreC and PostC have identified a number of signal transduction pathways conveying the cardioprotective signal from the sarcolemma to the mitochondria, some of which overlap for PreC and PostC. In fact, both PreC and PostC induce activation of signaling elements during the early reperfusion following the index ischemia[91] (Figure 2). Great attention has focused on the cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)-pathway[92-94], on the Reperfusion Injury Salvage Kinases (RISK) pathway[95,96], which involves the kinases Akt and ERK1/2, and more recently on the Survivor Activating Factor Enhancement (SAFE) pathway that has been suggested to contribute to PostC protection through the activation of tumor necrosis factor (TNF)-α, its receptor type 2, Janus kinase (JAK) and signal transducer and activator of transcription (STAT)-3[78,97]. All these pathways in preconditioning and postconditioning converge on the mitochondria *via* the modulation of several kinases including glycogen synthase kinase-3β, Bax/Bad and the ε isoform of protein kinase C (PKC) (for reviews see[10,78]). The modalities of mitochondrial control by cytosolic kinases depicted in Figures 2 and 3 are still controversial and are beyond the aims of this editorial.

Nevertheless, ROS signaling and acidosis in early reperfusion are two cardioprotective processes operating in early reperfusion in both preconditioning and postconditioning (Figures 2 and 3). They may act, first, directly on mPTP components to limit their opening and, then, may activate signaling pathways that have been suggested to converge again on mitochondria to decrease the susceptibility to mPTP opening and mediate protection (Figures 2 and 3). These two processes are discussed in the next sections.

ROS signaling and acidosis in early reperfusion

Before considering the beneficial role or ROS signaling, let us consider the forms and the detrimental effects of ROS within the heart.

Forms of ROS: ROS are generated in different cellular compartments and by several enzymes, including NADPH oxidases at the plasma membrane[98,99] and cytosolic xanthine oxidases[100]. Although ROS are produced by several extracellular and intracellular processes, in cardiomyocytes the mitochondria represent the most relevant site for ROS formation[101-105]. Within the mitochondria, most of the oxygen is reduced to water at respiratory complex IV. The mitochondrial formation of ROS might be modulated by $NO^{\bullet}$[106-108] which reversibly inhibits cytochrome oxidase[105,109-112]. This inhibition can be transformed into irreversible alterations of the respiratory chain when $NO^{\bullet}$ reacts with $O_2^{\bullet}$ to generate a large amount of peroxynitrite, which can produce the irreversible nitration of proteins[113]. Even nitric oxide synthases can become "uncoupled" resulting in the generation of $O_2^{\bullet}$ and $OH^{\bullet}$, instead of $NO^{\bullet}$ under certain conditions, such as scarcity of substrate and/or cofactors[112,113]. Apart from the electron transport chain, ROS can also be produced by monoamine oxidases (MAOs) in the OMM. MAOs transfer electrons from amine compounds to oxygen to generate hydrogen peroxide[102,114,115]. Within the mitochondria, p66Shc oxidizes reduced Cyt c, which induces the partial reduction of oxygen to peroxide[116-118].

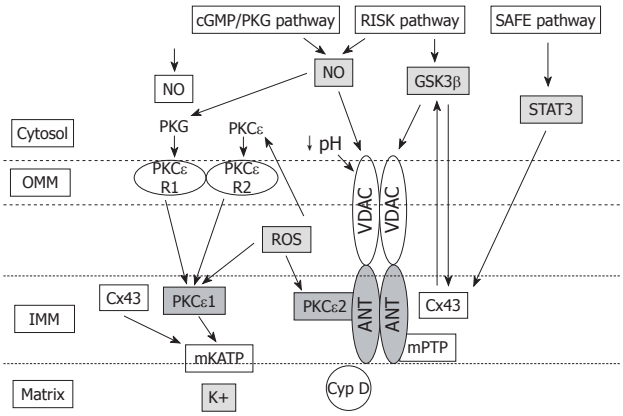

Figure 3 Extra- and intra-mitochondrial signalling: interactions among Cx43, mKATP, PKCε, ROS, and mPTP. The mPTP is believed to be composed of the adenine nucleotide transporter (ANT) in the inner membrane (IMM), the voltage-dependent anion channel (VDAC) of the outer membrane (OMM), and cyclophilin D (Cyp D) in the matrix. Signals arising from RISK and SAFE pathways are delivered to mitochondrial permeability transition pore (mPTP) *via* nitric oxide (NO)/glycogen synthase kinase 3 β (GSK3β) and signal transducer and activator of transcription 3 (STAT-3), respectively, and then to connexin 43 (Cx43) on the IMM. Signals arising from cGMP/PKG are delivered to mitochondria *via* the terminal protein kinase G (PKG). PKG phosphorylates an unknown OMM receptor, "R1", whereas PKCε act in conjunction to activate a distinct OMM receptor, "R2". These OMM receptors transmit the signal by an unknown mechanism to PKCε1 located at the IMM. The activated PKCε1 phosphorylates and opens mitochondrial ATP-sensitive potassium (mKATP). Also Cx43 located at the IMM regulated mKATP opening. The mKATP opening *via* PKCε1 causes K+ uptake and increased ROS production from respiratory chain. ROS produced by mKATP activation now diffuses and activates both PKCε1 and PKCε2 on the IMM and PKCε in the cytosol. ROS signalling may represent the link between mitochondria and cytosol. mPTP opening is prevented by cytosolic pH lowering, ROS signalling and PKCε2 activation.

Besides being a relevant site for ROS formation, mitochondrial function and structure are profoundly altered by oxidative stress[34], especially when mPTPs have long-lasting opening. In fact, mPTPs play a central role in the so-called ROS-induced ROS release (RIRR)[61,119,120]; excessive ROS facilitate mPTP opening which in turn favors ROS formation by inhibiting the respiratory chain because of mPTP-induced loss of Cyt c and pyridine nucleotides[12,34]. This vicious cycle is likely to be established at the onset of a rapid reperfusion when a large increase in ROS formation occurs along with pH recovery, and Ca^{2+} overload, thus inducing injury amplification, as discussed above and below (Figure 1).

Detrimental effects of excessive ROS: Various detrimental processes can result from an imbalance between the excess formation of ROS and limited antioxidant defenses (referred to as 'oxidative stress'). For instance, excessive ROS indiscriminately react with DNA, lipids and proteins[113,120-123]. The lack of protection of mitochondrial DNA by histones, the limited capacity of repair mecha-

nisms and the proximity of mitochondrial DNA to the production site of ROS by RIRR render the mitochondrial DNA highly susceptible to increased oxidative stress[124]. Excessive oxidative stress, besides contributing to irreversible myocardial injury, induces long-lasting mPTP opening leading to cellular dysfunction and cell death, and may also induce reversible injury during ischemia and reperfusion[105,125-128]. The reversible contractile dysfunction following myocardial ischemia/reperfusion ("stunning") is clearly a manifestation of excessive oxidative stress[129,130]. Whether stunning is due to RIRR has not yet been investigated.

On the other hand, as mentioned above, low levels of reactive species may act as secondary messengers, modulating cardioprotective signaling pathways by covalent modification of target molecules, referred to as 'redox signaling' or "ROS signaling", which is the main topic of the next section.

Beneficial effects of ROS signaling and acidosis - focus on early reperfusion

It has been proposed that reintroduction of oxygen after transient ischemia induces ROS production, but it does not protect against reperfusion injury because mPTPs open and trigger RIRR before the activation of endogenous survival pathways.

The key event for cardioprotection may be prolongation of cellular acidosis by cardioprotective phenomena during early reperfusion (Figure 2B). In fact acidic perfusion in early reperfusion or PostC, delaying pH normalization, could inhibit mPTP during the first minutes of reflow and allow for endogenous protective signaling pathways to be activated by ROS signaling. The delivery of oxygen during acidic perfusion or the brief intermittent reperfusions of PostC would promote mitochondrial ROS formation which has been proposed to activate isoforms of PKC through redox signaling[131]. Different isoforms of PKC appear as critical kinases in the signaling cascade leading to a reduced probability of mPTP opening after pH normalization[131-134] (see Figure 3 and below).

Not only mPTPs may be inhibited by a limited ROS production and acidosis, but also, as mentioned above, a transient or short duration opening of the mPTP has been suggested to induce a slight, transient formation of ROS that might be relevant for cardioprotection[51-54,56,57,135]. Supporting this concept, pharmacological and genetic inhibition of Cyp D was reported to attenuate both preconditioning-induced ROS formation and protection[136,137]. In brief, in protected reperfusion, low levels of ROS may act directly on mPTP components or activate signaling pathways that have been suggested to act on mitochondria decreasing their susceptibility to prolonged mPTP opening.

Redox signaling by transient/reduced formation of ROS is also included among the triggers of PostC[138]. In fact, we were the first to show that the ROS scavengers N-acetylcysteine (NAC)[132] and N-2-mercaptopropionyl glycine (MPG) prevent the protective effects of ischemic or pharmacological PostC[94,132,139,140], and the same ROS scavenger has been shown to block the protection afforded by acidic reperfusion[141]. In our laboratory isolated rat hearts were subjected to ischemia and reperfusion. While PostC significantly reduced infarct size, protection by PostC was lost in hearts perfused with NAC for the entire reperfusion period. Infarct size was still reduced when, in postconditioned hearts, perfusion with NAC was initiated after the first 3 min of reperfusion, demonstrating an essential role of ROS formation during early reperfusion in PostC protection[132]. Cardioprotection, induced with acidic buffer for the first 2 min of reperfusion, was blocked by MPG applied for 20 min in reperfusion[141], suggesting the involvement of ROS signaling in acidosis-induced protection. Infarct size reduction by either ischemic PostC, 1.4% isoflurane or 10 mg/kg of delta-opioid receptor agonist SNC-121 in mouse hearts *in vivo* were attenuated by the ROS scavenger MPG when administered a few minutes before but not 10 min after the postconditioning stimulus[140]. NAC in the first minutes of reperfusion also abolished postconditioning by bradykinin or sevoflurane in isolated rat hearts[94,139]. Importantly, in the human myocardium, desflurane-induced postconditioning was mediated by adenosine and bradykinin receptors *via* ROS signaling[115]. These studies support a central role for ROS signaling during early reperfusion in the protection by ischemic postconditioning and in the protection by acidosis in early reperfusion.

Mitochondrial ATP-sensitive potassium channels

Mitochondrial ATP-sensitive potassium (mK$_{ATP}$) channels in the mitochondrial inner membrane are considered targets of protective cascades, and play a pivotal role in ROS production, mainly superoxide anion derived from complex I of the electron transport chain[21,76,138,142,143]. Opening of the mK$_{ATP}$ channels and subsequent generation of ROS is considered to be a pivotal step in the mechanisms of pre- and postconditioning[3,21,76]. We found evidence that ROS signaling is downstream of mK$_{ATP}$ channel opening in isolated rat hearts subjected to ischemia/reperfusion with an intermittent infusion of diazoxide or diazoxide plus MPG at the onset of reperfusion, since MPG attenuated diazoxide-induced protection. However, while ROS scavenging attenuates infarct size reduction by postconditioning or diazoxide, increasing exogenous ROS formation with purine/xanthine oxidase at the onset of reperfusion does not confer protection[94]. It is likely that the type, the concentration, and/or the compartmentalization of reactive species may play a pivotal role in triggering protection at reperfusion. Nevertheless, we cannot rule out that a different ROS generator could trigger PostC protection.

In the context of cardioprotection, it has been reported that PKG- and/or Akt-dependent phosphorylation induces the opening of mK$_{ATP}$ promoting K^+ entry into mitochondria with consequent alkalinization of the mitochondrial matrix and generation of ROS with a protective signaling role. Indeed, PKG phosphorylates a protein on the OMM, which then causes the mK$_{ATP}$ on

the IMM to open. This implies that the protective signal is transmitted from the OMM to the IMM. This is accomplished by a series of intermembrane signaling steps that includes PKCε activation. The resulting ROS then activate a second PKC pool which, through another signal transduction pathway causes inhibition of the mPTP (the end effector) and reduction in cell death[3,76,144-146] (Figure 3). Pharmacological opening of mKATP channels by diazoxide contributes to the formation of small amounts of ROS[147]. Also, $NO^{\bullet}$ donors can activate mKATP channels in rabbit ventricular myocytes and can potentiate the protective effect of the mKATP opener diazoxide[143]. Besides cGMP/PKG-dependent phosphorylation, mKATP could be opened by direct reaction of $NO^{\bullet}$ and derivatives (S-nitrosylation), as well as by the action of H_2S *via* S-sulfhydration[4]. However, controversy exists on the nature, existence and opening of mKATP channels, which may also be a toxic process[145,148]. PKC activation leading to the opening of mKATP channels has been challenged by the Halestrap group: they demonstrated that preconditioning inhibits opening of the mPTP *in situ*, by an indirect mechanism probably involving decreased ROS production and Ca^{2+} overload at reperfusion[11,148].

Mitochondrial uncoupling

Mitochondrial uncoupling, i.e. proton influx into the mitochondrial matrix without phosphorylation of ADP, contributes to ROS formation[39,40]. Although controversy exists on the cardioprotective role of uncoupling, mild uncoupling secondary to activation of UCPs has been described to confer cardioprotection under several conditions, including myocardial reperfusion, likely by decreasing ROS production[41]. Intriguingly, it has been suggested that transient complex I inhibition during reperfusion is cardioprotective *via* attenuated ROS production[149,150]. Nevertheless, experimental evidence supporting the involvement of these changes in PostC protection has yet to be provided. Whether UCPs play a deleterious or protective role in ischemia tolerance is controversial[39-41].

Mitochondrial connexin-43

Mitochondrial connexin-43 (Cx43) has also been implicated in ROS-signaling, though its role is not completely defined[21,151,152]. Actually, within cardiomyocytes Cx43 is mainly localized at gap junctions, but it is also present in other organelle membranes, including the IMM of sarcolemmal mitochondria[152-154] where Cx43 regulates mitochondrial potassium flux[155,156]. It seems that Cx43 transolocates to the IMM, with cardioprotection *via* the intervention of heat shock protein 90-TOM (translocation of the outer membrane) import pathway[154].

Mitochondrial Cx43 has been described to be essential for preconditioning protection[154,157,158], but a recent study in mice heterozygous for Cx43 (Cx43+/-) indicates that it does not play a significant role in PostC protection. Actually, Cx43 is a target of several protein kinases, and mitochondrial Cx43 is highly phosphorylated under physiological conditions[159]; it seems that in the IMM of a subset of cardiomyocyte mitochondria, subsarcolemmal mitochondria, the phosphorylated portion of Cx43 increases with ischemia[152] and decreases with PostC[138]. Since a decrease in the mitochondrial Cx43 content is sufficient to abolish the cardioprotection by diazoxide preconditioning, i.e. reduces ROS formation[147], one can speculate that Cx43 reduction in PostC may be one of the mechanisms to reduce excessive ROS production in the reperfusion phase. Recently it has been suggested that genetic ablation or pharmacological inhibition of mitochondrial Cx43 confers resistance to mKATP channel opening in response to diazoxide in patch-clamped mitoplasts (mitochondria devoid of the OMM). However, the open-probability of the mKATP channel was not affected under baseline conditions; thus it is likely that Cx43 regulates this channel activity rather than constituting the pore forming unit of the mKATP channel[156].

Timing and targets of ROS signaling in cardioprotection

In the context of cardioprotection, reactive species with a signaling role are suggested to be formed during three time points: (1) during preconditioning-ischemia and/or (2) during reperfusion that follows the brief preconditioning-ischemia; and (3) in the initial part of reperfusion that follows the index ischemia; both in the postconditioning and in the preconditioning context.

During preconditioning-ischemia: A wide body of evidence exists demonstrating that appreciable formation of ROS occurs during ischemia[126,128,160-162]. In fact, mitochondrial ROS formation is favored by a decrease in electron flow resulting from respiratory chain inhibition, and is counteracted by uncoupling that is generally produced by an increased IMM permeability to protons. Therefore, the inhibition of the electron transport chain caused by insufficient oxygenation facilitates the escape of electrons that can react directly with the scarce available oxygen resulting in ROS formation.

During reperfusion that follows brief preconditioning-ischemia: Small amounts of ROS may be formed during reperfusion following a short period of preconditioning ischemia. To support this viewpoint, the group of Downey[163] used MPG (a cell-permeant ROS scavenger[164]) to test whether the ROS that triggers protection are produced during the ischemic or the reperfusion phases of the preconditioning maneuvers. These authors concluded that protective redox signaling occurs when molecular O_2 is reintroduced following the brief preconditioning coronary occlusion.

In the initial part of reperfusion that follows the index ischemia in postconditioning: The above observations were done in the preconditioning phase, i.e. before the index ischemia, and extended to PostC itself. In fact, as reported above, the protective effect of PostC was attenuated by infusing, during early reperfusion, large spectrum ROS scavengers, making the oxygenated perfusate

alkaline during the early reperfusion phases or making the early reperfusion buffer hypoxic[50,94,131,132,142,165].

Early reperfusion events in preconditioned heart: Clearly, both PreC and PostC, besides sharing a number of signaling elements, induce activation of signaling elements (RISK and SAFE) during early reperfusion following the prolonged index ischemia[78,91,166] (Figure 2). It is now thought that, after a triggering phase in the preischemic period, the actual protection by PreC occurs in the reperfusion rather than in the ischemic phase, with the repopulation of sensitized G-protein-coupled receptors at the beginning of myocardial reperfusion following the index ischemia[167]. Hence, reintroduction of O_2 at the beginning of reperfusion permits generation of signaling ROS, which will activate the PKC-dependent signaling cascade. In fact the PreC protective effect was also attenuated by infusing. during early reperfusion. large spectrum ROS scavengers, making the oxygenated perfusate alkaline during the early reperfusion phase or making the early reperfusion buffer hypoxic[56,166,167].

Targets of ROS signaling: Reactive species function as trigger molecules of protection by activating protein kinases such as PKC within and outside the mitochondria[3,146,147,168,169], as well as the mitogen-activated protein kinase p38 and/or JAK/STAT[170,171]. Several mitochondrial components are targeted by reactive species *via* oxidative/nitrosative processes[4,10]. Accordingly, many large spectrum scavengers of ROS, such as ascorbic acid, MPG or NAC, attenuate infarct size reduction by ischemic or pharmacological PreC or PostC, in several animal species[2-4,132,145,169,172]. Since a target of ROS in redox signaling is the PKC, hearts can be preconditioned by simply infusing free radicals into the coronary arteries, and that protection can be blocked by a PKC antagonist[76,167]. Evidence exists that ROS-activated PKC will also protect the reperfused heart[132,169]. This sequence would explain the observation that a PKC activator could rescue hearts experiencing acidic and hypoxic reperfusion[131]. Moreover, chelerythrine, a non-specific PKC antagonist, blocks PostC protection[132-134].

Indeed, it has been reported that ROS can activate PKC *in vitro* by reacting with thiol groups associated with the zinc finger region of the molecule[173]. Reactive nitrogen species-dependent activation of PKC, possibly *via* a redox-sensitive S-nitrosylation process, has been also suggested; a process which also occurs within mitochondria[4,174,175]. Recently, we have observed in an *ex vivo* study that PostC induces downregulation of superoxide dismutase (SOD), whereas catalase activity does not change in the early reperfusion phase. Moreover, PostC reduces 3-nitrotyrosine and increases S-nitrosylated protein levels, thus contributing to cardioprotection triggering[176]. The persistence of acidosis[50,68,131,141,165] and the $NO^{\bullet}$ augmentation (enzymatic and non-enzymatic production)[132] in early reperfusion of postconditioned hearts, together with SOD downregulation may favor nitrosylation and/or may limit protein de-nitrosylation. In fact SOD is a de-nitrosylating enzyme[177]. Intriguingly, exogenous-SOD prevents PostC-triggering, whereas exogenous-catalase does not interfere with PostC protection. That is, the addition of exogenous-SOD during PostC maneuvers does not allow the early reduction in overall SOD activity, usually induced by PostC.

Preservation of functional and morphological integrity of mitochondria

Preconditioning and postconditioning activate cardioprotective pathways that are protective against reperfusion injury *via* preservation of the functional and morphological integrity of mitochondria. The protection of PostC against apoptosis is mediated by reduced generation of superoxide anions, lowered activity of c-Jun-N-terminal kinases/p38, lowered levels of caspases 3 and 8, reduced release of TNFα, and by the modulation of the Bax/Bcl-2 ratio[178]. PostC increases the levels of anti-apoptotic markers, including the cardioprotective kinase Pim-1, decreases pro-apoptotic markers, e.g. Cyt c, and preserves the mitochondrial structure. In fact, at the onset of reperfusion, mitochondria undergo profound structural alterations. In particular, post-ischemic mitochondria are characterized by disruption of membranes, broken christae and the appearance of dense granules within the mitochondrial matrix, which are caused by massive accumulation of Ca^{2+}, generating insoluble calcium phosphate precipitate[179]. These mitochondrial damages are reduced by PostC[138]. Carbonylation of mitochondrial proteins was prevented and aconitase activity was preserved in the PostC hearts suggesting that mitochondrial integrity was associated with a diminution in oxidative stress[180]. However, PostC does not influence mitochondrial respiration[181]. In particular, PostC does not affect basal state 4 or ADP-stimulated state 3 respiration, excluding uncoupling or inhibition of the respiratory chain as a mechanism of mPTP inhibition[182]. Nevertheless, while basal respiration was not affected, ADP-stimulated respiration was increased after pharmacological PostC with morphine[183]. This is in line with many reports showing that a mild degree of mitochondrial dysfunction confers protection against ischemia/reperfusion injury[175,184].

In summary, ROS signaling before index ischemia, i.e. during brief preconditioning ischemia and/or during the following reperfusion, is clearly involved in the triggering of preconditioning protection. Excessive ROS formation during reperfusion, following infarcting ischemia, enhances cell death, but ROS signaling during early reperfusion is essential for protection of ischemic and some pharmacological preconditioning and postconditioning against reperfusion injury. In early reperfusion, opening of mK_{ATP} channels may be upstream of ROS signaling. Cardioprotective procedures delay the post-ischemic recovery of intracellular pH that may prevent mPTP opening directly and indirectly (i.e. by inhibiting calpain activation). In addition, mPTP opening may be further prevented by a ROS signaling that appears to depend on acidosis and by a decrease in intracellular Ca^{2+}. ROS signaling triggers a protective kinase cascade starting from PKC and converging on mPTPs.

Thus, mPTP closure may be dependent on ROS signaling effects, both upstream, together with an acidotic effect, and downstream, dependent on kinase effects. Therefore, mitochondria are involved in at least four different steps to limit reperfusion injury: (1) as the target of acidosis (i.e. prevention of mPTP opening); (2) as triggers or signal amplifiers (i.e. activation of mKATP channels and resulting formation of small amounts of ROS); (3) as the target of signaling pathways and end-effectors (i.e. inhibition of mPTP opening and of release of pro-apoptotic factors into the cytosol); and (4) as targets of damage and protection from it (i.e. functional and morphological integrity).

THERAPEUTIC IMPLICATIONS

The signaling role of ROS in early reperfusion must be kept in mind for successful treatment in reperfusion. Clearly, the mPTP is a major factor in determining cell death, and mPTP inhibition affords significant cardioprotection[12,32,185,186], a concept that has been successfully translated into the clinical setting[81,82,187,188]. In particular, postconditioning transition to the clinical setting has proven the existence of lethal myocardial reperfusion injury in man, and the clinical studies suggest that 40%-50% of the final reperfused infarct in humans may be attributable to myocardial reperfusion injury[189].

In AMI patients the involved coronary artery may be opened by either angioplasty or thrombolysis, with or without application of a stent. The ischemic postconditioning, though reserved for patients reperfused by primary angioplasty, may provide impressive results when the no-reflow phenomenon, the infarct size and the myocardial contractile function are considered, raising great hopes for potential clinical benefits. Feasible in every patient, the pharmacological postconditioning, including CsA infusion, would allow the expansion of postconditioning protection to almost all ST-elevation AMI patients. Obviously restoring reperfusion to the ischemic myocardium is the definitive strategy in reducing infarct size. However, blood flow may not be restored to all segments of the microvasculature in the post-ischemic myocardium[190], a situation that is associated with the no-reflow phenomenon as a predictor of adverse long-term outcomes in patients[191]. The obvious implication of low- or no-reflow is that the blood supply is inadequate to sustain contractile function, and the decrease may be severe enough to induce cell death of the involved myocardium. Reducing the no-reflow area may lead to smaller infarcts, less adverse remodeling and less severe heart failure. Post-ischemic blood flow in a small group of patients undergoing PCI for AMI was studied by Laskey[192]. Patients were assigned to receive standard care *vs* an "ischemic conditioning" stimulus. While the control group (standard care) had a 90-s balloon inflation only before withdrawal of the catheter, the conditioned group had a 90-s inflation followed by 3-5 min of balloon deflation, and after that the balloon was advanced distal to the stenosis. These conditioned patients have shown an improved post-ischemic coronary blood flow as revealed by an increased peak coronary blood flow velocity, diastolic/systolic velocity ratio and blood flow velocity reserve (evaluated with Doppler flow wire) compared to the control group. Staat *et al*[81] used blush grade, the speed by which contrast is washed out of the myocardium at risk, as a marker of myocardial reperfusion after the initial period of reflow in patients subjected to standard angioplasty or postconditioning, which consisted of four cycles of 60-s deflation/inflation of the angioplasty balloon in the target vessel. In postconditioned patients the blush grade was 25% greater than that of control patients. Also Ma *et al*[193] found that post-ischemic coronary blood flow in the target vessel was greater in AMI patients who received postconditioning treatment. This was associated with lower markers of lipid peroxidation by ROS and lower plasma levels of myocardial creatine kinase. Moreover, brachial arterial endothelium-mediated (flow-dependent) relaxation in response to transient cuff occlusion applied 24 h after PCI was better in the postconditioned patients. Although flow-dependent vasodilator effects in the brachial artery do not directly reflect physiology of the coronary vascular endothelium, it may be reflective of a "remote" protection to the endothelium of other organs, which then becomes a surrogate measure of the coronary vascular endothelium. Actually remote ischemic postconditioning (conditioning stimuli applied to a distant organ during reperfusion of the target organ) induced by transient episodes of ischemia of distant organs, including kidney, arms and legs, is a clinically feasible method for protecting the heart against injury at the time of reperfusion. Recently, it has been observed in rats that remote ischemic perconditioning may reduce infarct size, and that repeated remote postconditioning further reduces adverse remodeling of the left ventricle and may improve survival in a dose-dependent fashion[194]. Indeed, remote postconditioning has been reported experimentally[194,195], and correlated with endothelial protection in humans[196,197]. Thus, although these data suggest that local and remote ischemic postconditioning have favorable effects on recovery of microvascular perfusion following relief of ischemia, further experimental and clinical studies are needed to establish whether postconditioning attenuates microvascular injury and the extent of no-reflow.

As mentioned above, a pharmacological approach may be more suitable. In fact, initial progress has been made with novel approaches for preventing myocardial reperfusion injury by administering drugs in the first minutes of reperfusion; preliminary clinical data indicate that drugs targeting mPTPs or RISK may confer benefits to patients with AMI, with and without comorbidities, above that provided by myocardial reperfusion alone. Very good results are obtained with drugs such as CsA as an mPTP desensitizer[81,187,188], as well as with other drugs targeting RISK, such as erythropoietin and its analogs[198,199]. Importantly, similar cardioprotective effects were obtained with other drugs acting on mPTP, confirming the relevance of this approach. For instance, derivatives of CsA, such as [N-methyl-ala^6]CsA, [N-methyl-Val^4]CsA, Debio 025,

NIM811, or sanglifehrin A[11,12,56,58,59,68,71,136,189] also prevented myocardial ischemia/reperfusion injury in an experimental setting. Importantly, in small, proof-of-concept trials[81,82,187,189], the administration of CsA in patients with AMI at the time of reperfusion has been associated with smaller infarct size. The efficacy of treatment has been assessed measuring the release of the cardiac biomarkers creatine kinase and troponin I and by measuring the area of late hyperenhancement of the reperfused myocardium on magnetic resonance imaging (MRI). In one of this studies[187], the area under the curve for the creatine kinase concentration suggested that the administration of cyclosporine induced a reduction in infarct size of approximately 40%. This result was confirmed by a reduction in the area of late hyperenhancement on MRI. However, the area under the curve for the troponin I concentration was not significantly reduced by the administration of cyclosporine. Mewton *et al*[188] recently examined whether CsA modified left ventricle remodeling in patients. Cardiac MRI was performed at day 5 and after 6 mo. The authors reported that CsA did not exert any deleterious effect on left ventricle remodeling, and confirmed that infarct size reduction persisted after 6 mo. In addition, infarct size reduction by CsA was associated with a lower left ventricle dilatation at day 5, which was maintained at 6 mo. These data require confirmation in larger clinical trials.

CONCLUSION

It appears that many different signals can induce (and inhibit) mPTP formation, strictly linking ischemia/reperfusion stress and damage to the mitochondria. This highlights the capacity of mitochondria to function as general cell death sensors and to integrate many lethal signals. Clearly, mitochondria and ROS are attractive mechanistic targets for cardioprotection. Indeed, proof-of-concept studies demonstrated beneficial effects of the mPTP desensitizer CsA during early reperfusion in patients with AMI. Patient-tailored treatment to either prevent mPTP formation or the upstream events leading to mPTP opening may be achievable in the next future.

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